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Are Putative Periodontal Pathogens Reliable Diagnostic Markers?▽

Birgit Riep,[1] Lilian Edesi-Neuß,[2,3] Friderike Claessen,[3] Horst Skarabis,[4] Benjamin Ehmke,[5] Thomas F. Flemmig,[6] Jean-Pierre Bernimoulin,[2] Ulf B. Göbel,[3] and Annette Moter[3]*

Abteilung für Zahnerhaltung und Parodontologie,[1] Abteilung für Parodontologie und Synoptische Zahnmedizin,[2] and Institut für Mikrobiologie und Hygiene,[3] Charité-Universitätsmedizin Berlin, Berlin, Germany; Institut für Statistik, Philosophie und Sozialwissenschaften, Freie Universität Berlin, Berlin, Germany[4]; Poliklinik für Parodontologie, Westfälische Wilhelms Universität Münster, Münster, Germany[5]; and Department of Periodontics, School of Dentistry, University of Washington, Seattle, Washington[6]

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Periodontitis is one of the most common chronic inflammatory diseases. A number of putative bacterial pathogens have been associated with the disease and are used as diagnostic markers. In the present study, we compared the prevalence of oral bacterial species in the subgingival biofilm of generalized aggressive periodontitis (GAP) (n = 44) and chronic periodontitis (CP) (n = 46) patients with that of a periodontitis-resistant control group (PR) (n = 21). The control group consisted of subjects at least 65 years of age with only minimal or no periodontitis and no history of periodontal treatment. A total of 555 samples from 111 subjects were included in this study. The samples were analyzed by PCR of 16S rRNA gene fragments and subsequent dot blot hybridization using oligonucleotide probes specific for *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, a *Treponema denticola*-like phylogroup (Treponema phylogroup II), *Treponema lecithinolyticum*, *Campylobacter rectus*, *Fusobacterium* spp., and *Fusobacterium nucleatum*, as well as *Capnocytophaga ochracea*. Our data confirm a high prevalence of the putative periodontal pathogens *P. gingivalis*, *P. intermedia*, and *T. forsythia* in the periodontitis groups. However, these species were also frequently detected in the PR group. For most of the species tested, the prevalence was more associated with increased probing depth than with the subject group. *T. lecithinolyticum* was the only periodontopathogenic species showing significant differences both between GAP and CP patients and between GAP patients and PR subjects. *C. ochracea* was associated with the PR subjects, regardless of the probing depth. These results indicate that *T. lecithinolyticum* may be a diagnostic marker for GAP and *C. ochracea* for periodontal health. They also suggest that current presumptions of the association of specific bacteria with periodontal health and disease require further evaluation.

Periodontitis is a chronic inflammatory disease of infectious origin leading to destruction of tooth-supporting tissues and is the major cause of tooth loss in adults. However, all patients are not equally susceptible to periodontitis. In addition to the microbial challenge, other factors, such as genetics, environment, and host factors, play a role in the pathogenesis of this disease (24). The most common form is chronic periodontitis, which is characterized by a slow, gradual loss of periodontal attachment (3). In contrast to this form, rapid destruction of periodontal attachment is evident in aggressive periodontitis. Generalized aggressive periodontitis usually affects young adults under the age of 30, with attachment loss occurring in pronounced episodes of tissue destruction (2).

Many studies have been performed to evaluate the composition of the subgingival biofilm and identify key periodontal pathogens by both cultivation and molecular methods. More than 700 different species have been identified in the oral cavity, many of which are yet to be cultivated (1, 25). Of these species, only a small number are suspected periodontal pathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. This group of bacteria, characterized as the "red complex," was highly associated with periodontal tissue destruction (28). Other bacteria, such as *Prevotella intermedia*, *Campylobacter rectus*, *Fusobacterium* species, *Peptostreptococcus micros*, and various spirochetes, have been implicated in the development of periodontitis (33). Aggressive periodontitis has been postulated to be frequently associated with *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* (2). Localized aggressive periodontitis in particular seems to be characterized by specific infection with *A. actinomycetemcomitans* (27, 34), whereas chronic periodontitis is rather a mixed bacterial infection, not associated with any specific microorganism.

In the present study, we reevaluated the association of putative periodontal pathogens in patients with generalized aggressive or chronic periodontitis versus species in a periodontitis-resistant control group to identify species which are incompatible with periodontal health. As a control group, we chose a population of older adults with only minimal or no periodontitis and no history of periodontal treatment, considering these subjects resistant to periodontitis. We analyzed the presence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, a *T. denticola*-like phylogroup (Treponema phylogroup II), *Treponema lecithinolyticum*, *C. rectus*, *Fusobacterium* spp., and *Fusobacterium nucleatum*, as well as *Capnocytophaga ochracea*. We also examined whether the presence or absence of these species was related to pocket depth.

MATERIALS AND METHODS

Clinical samples. Patients diagnosed with chronic (CP) or generalized aggressive periodontitis (GAP) according to the criteria of the 1999 International

* Corresponding author. Mailing address: Charité-Universitätsmedizin Berlin, Institut für Mikrobiologie und Hygiene, Dorotheen-Str. 96, D-10117 Berlin, Germany. Phone: 49 30 450524226. Fax: 49 30 450524902. E-mail: annette.moter@charite.de.

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TABLE 1. Clinical criteria for patient selection

Group	Criterion(a) for inclusion
PR control	Age of ≥65 yr ≥20 natural teeth Probing depth at any site of ≤5 mm Clinical attachment loss at any site of ≤2 mm
CP	≥4-mm probing depth at ≥30% of residual teeth
GAP	Disease onset estimated at <30 yr based on clinical examination, past radiographs, and/or interview ≥6-mm probing pocket depth at >3 permanent teeth other than first molars and incisors

TABLE 2. Patient demographics

Characteristic	Value for group[a]		
	GAP	CP	PR
Age (yr) ± SD	34.4 ± 6.5	55.2 ± 11.2	66.6 ± 1.5
Gender [no. (%)]			
Male	19 (43.2)	21 (45.7)	8 (38.1)
Female	25 (56.8)	25 (54.3)	13 (61.9)
Smoker [no. (%)]			
Current	17 (38.6)	15 (32.6)	ND[b]
Former	4 (9.1)	4 (8.7)	ND
Never	23 (52.3)	27 (58.7)	ND
Patient samples			
Mean PD[c] (mm) ± SD	7.5 ± 2.9	5.2 ± 2.4	3.7 ± 0.9

[a] For GAP, $n = 44$; for CP, $n = 46$; for PR, $n = 21$.
[b] ND, not determined.
[c] PD, probing depth.

Workshop for Classification of Periodontal Disease and Conditions (3) were included in this study. Patients with GAP ($n = 44$) and with CP ($n = 46$) had been referred for periodontal treatment to the Departments of Periodontology at the University Hospital Charité Berlin and the University of Würzburg, respectively. A periodontitis-resistant (PR) group of 21 subjects 65 years of age or older with minimal or no periodontitis recruited from a private practice in Berlin served as the control group. Clinical criteria for patient selection are presented in Table 1. All subjects were previously untreated. Exclusion criteria for all subjects were chronic systemic disease or anti-inflammatory or antimicrobial therapy within the last 6 months; pregnant or lactating women were also excluded. Demographics of all subjects are presented in Table 2.

A total of 555 samples from 111 subjects were included in this study after informed consent was obtained. For each subject, subgingival plaque samples were taken from the four deepest periodontal pockets and if present from an additional healthy control site with a probing depth of ≤3 mm by insertion of three sterile paper points (ISO 35; Becht, Offenburg, Germany) after removal of supragingival plaque. In the GAP group all sample sites with the deepest periodontal pockets showed a probing depth of ≥6 mm and in the PR group a probing depth of <6 mm. The paper points were removed after 10 s, placed in 1 ml of reduced transport fluid (30) containing 25% glucose, transferred to the laboratory, and processed immediately.

DNA extraction and amplification. PCR amplification of the subgingival plaque samples was performed as described earlier (20). Briefly, aliquots (100 μl) of each specimen were centrifuged at 13,000 × *g* for 10 min in a Labofuge 400 R instrument (Heraeus, Germany). The pellets were resuspended in 100 μl lysis buffer (5). No further purification of nucleic acids was performed, and 1 μl of the bulk DNA was used for in vitro amplification by PCR (final reaction volume, 100 μl) in a thermal cycler (Trioblock, Biometra, Germany), using 30 cycles of denaturation (1 min, 95°C), annealing (1 min, 56°C), and extension (1 min, 72°C). The broad-range bacterial primers TPU1 (5′-AGA GTT TGA TCM TGG CTC AG-3′) (corresponding to positions 8 to 27 in the *Escherichia coli* 16S rRNA gene) (4) and RTU3 (5′-GWA TTA CCG CGG CKG CTG-3′) (corresponding to complementary positions 519 to 536 in *E. coli* 16S rRNA) (4) were used for 16S rRNA gene amplification. Successful amplification was verified by agarose gel electrophoresis.

Oligonucleotide probes. Species-specific, genus-specific, or phylotype-specific oligonucleotide probes (16 to 29 bases) as 16S rRNA/DNA-directed probes were used to detect *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema* group II (TRE II) (*Treponema denticola*-like), *Treponema lecithinolyticum*, *Campylobacter rectus*, *Capnocytophaga ochracea*, *Fusobacterium* spp., and *F. nucleatum*.

Previously published oligonucleotides were reevaluated. In order to assess the specificity, the target sequences were compared to 16S rRNA entries of prokaryotes in the EMBL and GenBank databases, accessible (as of July 2002) by using the software program BLASTN of the Husar program package (version 4.0; Heidelberg Unix Sequence Analysis Resources; DKFZ, Heidelberg, Germany). All probes were checked with the program OLIGO 4.0 for their use in a hybridization assay. Oligonucleotide probe sequences and references are shown in Table 3. More details on the oligonucleotide probes are available at probeBase (16).

Bacterial strains. Bacterial strains were used as positive and negative controls (Fig. 1). The identities of target bacteria and closely related species were verified by 16S rRNA gene sequencing or biochemical tests using the rapid ID32A system (bioMérieux, Marcy-l'Etoile, France). PCR analysis of the reference bacteria was performed with cell pellets collected from culture as described for the subgingival plaque samples.

Dot blot hybridization. Amplified DNA was spotted onto nylon membranes, and dot blot hybridization was carried out as described earlier (20). Briefly, an aliquot (1 μl) of heat-denatured PCR product was applied on nylon membranes (Hybond N; Amersham, Buckinghamshire, Great Britain) and fixed by UV cross-linking.

PCR products of 42 amplified DNA samples of oral and extraoral bacterial strains served as controls to ensure stringent hybridization conditions in all dot blot hybridizations. These included the respective species as positive controls and the phylogenetically closest relatives at the probe binding sites. Furthermore, if the probes TRE II or TLEC were used, a control membrane with amplified DNA samples from either recombinant clones retrieved from the original oral treponema 16S rRNA gene library (5) or known cultivable treponemes was included as a control in all dot blot hybridizations as published previously (21).

Probes were labeled with digoxigenin-ddUTP (Boehringer, Mannheim, Germany) and detected by chemiluminescence according to the manufacturer's recommendations. All hybridizations were performed at 54°C. Stringency washes were optimized for each probe by varying the washing temperature (40°C to 64°C) and the washing buffer (containing 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.2% sodium dodecyl sulfate [SDS] or 0.1× SSC–0.1% SDS). X-ray films were exposed to the membranes for 2 to 12 h. After stripping with 0.2 N NaOH with 0.1% SDS (stripping buffer), identical membranes were used for multiple hybridization experiments with the probes mentioned above.

Statistical analysis. Statistical evaluation was performed for descriptive and for inferential purposes. The exact chi-square tests with Bonferroni's correction for multiple comparisons were applied to compare the prevalences of the target bacteria between the three groups. A subject was regarded as positive for a certain genospecies if the organism was detected in at least one sample. The Bonferroni-corrected Mann-Whitney U tests, as well as tests for a difference in proportions using the central limit theorem, were used to examine differences in the numbers of sites per patient positive for the target species. Analysis of variance with Bonferroni's correction for multiple comparisons in the post hoc tests was performed for determination of differences between the groups with respect to target bacteria at different probing depths. The mean percentages of positive sites were derived by averaging the positive sites of each species within a subject and then across subjects in the clinical groups. The presence of bacteria at various probing depths was evaluated by logistic regression analysis adjusted for clustering on the subjects. For all statistical tests, P values less than 0.05 were considered significant.

RESULTS

PCR amplification was successful for all subgingival plaque specimens. In dot blot hybridization, none of the negative controls showed a signal. This indicated that no carryover of amplified material occurred (data not shown). Using optimized hybridization and washing conditions for each oligonucleotide probe, only PCR products of the appropriate target species

TABLE 3. Oligonucleotide probes used for dot blot hybridization

Target species/genus	Probe name as deposited in probeBase	Probe sequence (5′–3′)	Reference
A. actinomycetemcomitans	ACAC	TCC ATA AGA CAG ATT C	Sunde et al., 2003 (29)
P. gingivalis	POGI	CAA TAC TCG TAT CGC CCG TTA TTC	Sunde et al., 2003 (29)
P. intermedia	PRIN	CTT TAC TCC CCA ACA AAA GCA GTT TAC AA	Sunde et al., 2003 (29)
T. forsythia	B(T)AFO	CGT ATC TCA TTT TAT TCC CCT GTA	Sunde et al., 2003 (29)
T. denticola-like (TRE II)	TRE II	GCT CCT TTC CTC ATT TAC CTT TAT	Moter et al., 1998 (20)
T. lecithinolyticum	TLEC	CAC TCT CAG AAA GGA GCA AGC TCC	Moter et al., 2006 (21)
C. rectus	CARE	TTA ACT TAT GTA AAG AAG	This study
C. ochracea	CAOC	TCG GGC TAT CCC CCA GTG AAA GGC AGA T	This study
Fusobacterium spp.[a]	FUSO	CTA ATG GGA CGC AAA GCT CTC	Sunde et al., 2003 (29)
F. nucleatum *F. periodonticum*	FUNU	ATG TTG TCC CTA V(GCA)CT GTG AGG C	This study

[a] The sequence of the genus *Fusobacterium*-specific probe matches those of *F. nucleatum*, *F. necrophorum*, *F. mortiferum*, *F. simiae*, *F. gonidiaformans*, *F. alocis*, *F. varium*, *F. russii*, *F. ulcerans*, *F. periodonticum*, *F. perfoetens*, *F. equinum*, *F. naviforme*, *and F. canifelinum.*

were detected by the specific probes on the control membranes. No cross-reactions were observed.

Prevalences of target bacteria in subject groups. All target species were detected in subgingival plaque samples from both periodontitis groups and the PR group. Obvious differences were observed between the patient groups regarding signal intensities of the dot blot hybridizations, suggesting a higher number of periodontal pathogens in the GAP patients (Fig. 1). However, since PCR-amplified DNA was used for hybridization experiments, the signal intensity was not quantitatively analyzed.

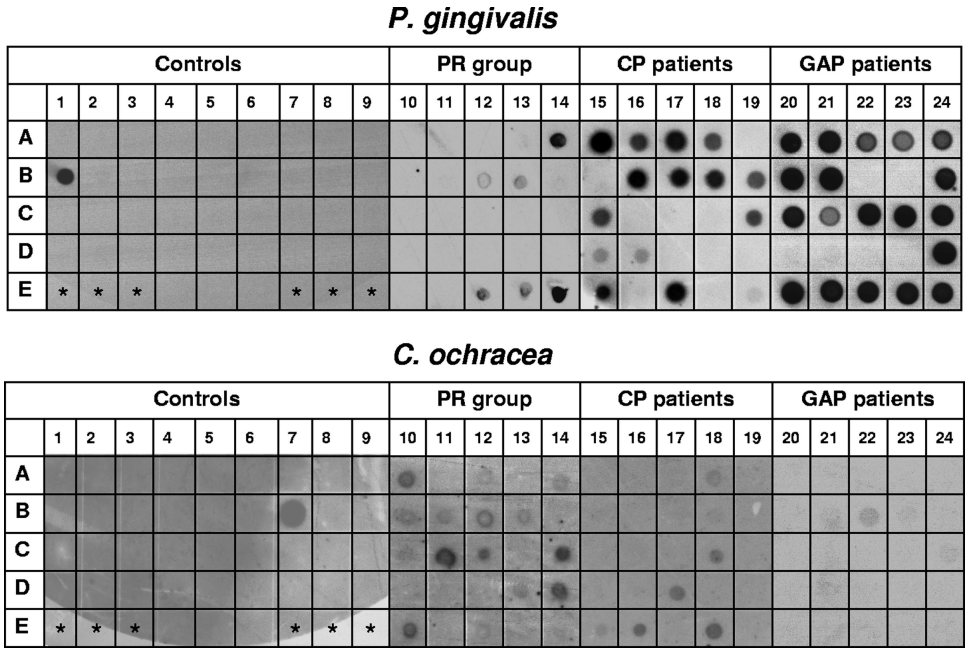

FIG. 1. Dot blot hybridizations of identical membranes with probes for *P. gingivalis* and *C. ochracea*. In columns 1 to 9, PCR products of the following strains were applied as controls: *Actinobacillus actinomycetemcomitans* ATCC 43718 (A1); *Actinobacillus actinomycetemcomitans* ATCC 33384 (A2); *Actinobacillus actinomycetemcomitans* serotype a (A3); *Leptotrichia buccalis* MCCM 00448 (A4); *Pasteurella haemolytica* ATCC 33396 (A5); *Haemophilus influenzae* ATCC 33391 (A6); *Haemophilus influenzae* clinical isolate (A7); *Haemophilus aphrophilus* NCTC 55906 (A8); *Haemophilus paraphrophilus* ATCC 29241 (A9); *Porphyromonas gingivalis* ATCC 33277 (B1); *Prevotella intermedia* ATCC 25611 (B2); *Porphyromonas assacharolyticus* ATCC 25260 (B3); *Prevotella nigrescens* NCTC 9336 (B4); *Prevotella oralis* MCCM 00684 (B5); *Prevotella buccalis* ATCC 33690 (B6); *Capnocytophaga ochracea* ATCC 27872 (B7); *Capnocytophaga sputigena* ATCC 33612 (B8); *Capnocytophaga gingivalis* ATCC 33624 (B9); *Campylobacter rectus* ATCC 33238 (C1); *Campylobacter concisus* ATCC 33237 (C2); *Bacteroides gracilis* ATCC 33236 (C3); *Bacteroides fragilis* ATCC 25285 (C4); *Eikenella corrodens* CCUG 2138 (C5); *Kingella kingae* ATCC 23330 (C6); *Veillonella parvula* ATCC 10790 (C7); *Veillonella dispar* ATCC 17748 (C8); *Klebsiella pneumoniae* ATCC 23357 (C9); *Fusobacterium nucleatum* ATCC 25586 (D1); *Flavobacterium odoratum* MCCM 02932 (D2); *Neisseria lactamica* ATCC 23970 (D3); *Streptococcus mutans* ATCC 35668 (D4); *Streptococcus intermedius* ATCC 27335 (D5); *Actinomyces viscosus* ATCC 15987 (D6); *Actinomyces israelii* ATCC 10048 (D7); *Eubacterium lentum* ATCC 25559 (D8); *Selenomonas* sp. clinical strain (D9); *Fusobacterium simiae* CCUG 16798 (E4); *Fusobacterium periodonticum* CCUG 14345 (E5); and *Fusobacterium necrophorum* NCTC 25286 (E6). Asterisks indicate empty fields without PCR product. In columns 10 to 14, 15 to 19, and 20 to 24, PCR products from subgingival plaque samples of PR subjects, CP patients, and GAP patients (five patients each) were applied, respectively. Each column represents four deep pockets plus one control site from a single patient. For an accurate analysis, the samples from each patient were spotted on the membrane in a random order. Samples were considered positive if the dot was clearly visible above the background level of the negative controls.

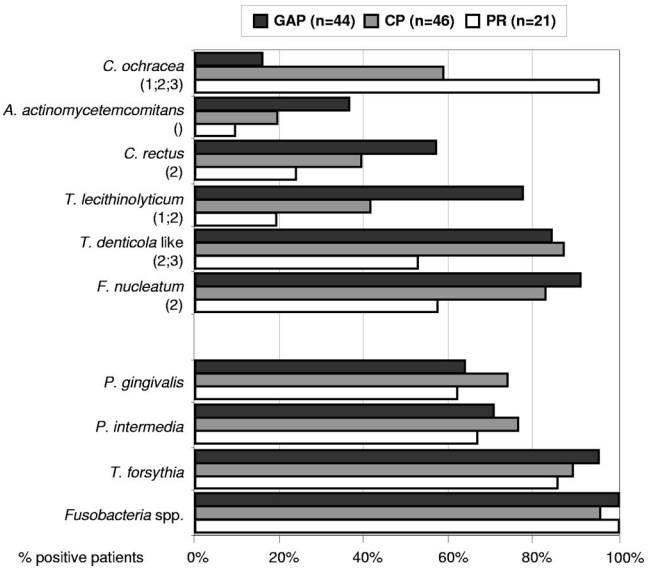

FIG. 2. Prevalence of target species in the GAP patients, CP patients, and PR subjects as determined by dot blot hybridizations using oligonucleotide probes. A patient was regarded as positive if at least one sample was positive. Numbers in parentheses indicate statistical significances between the groups: 1, GAP versus CP; 2, GAP versus PR; and 3, CP versus PR, as determined by chi-square analysis with Bonferroni's correction for multiple comparisons. Empty parentheses (), as for *A. actinomycetemcomitans*, indicate an overall significance but no significant differences between the groups in the post hoc test results.

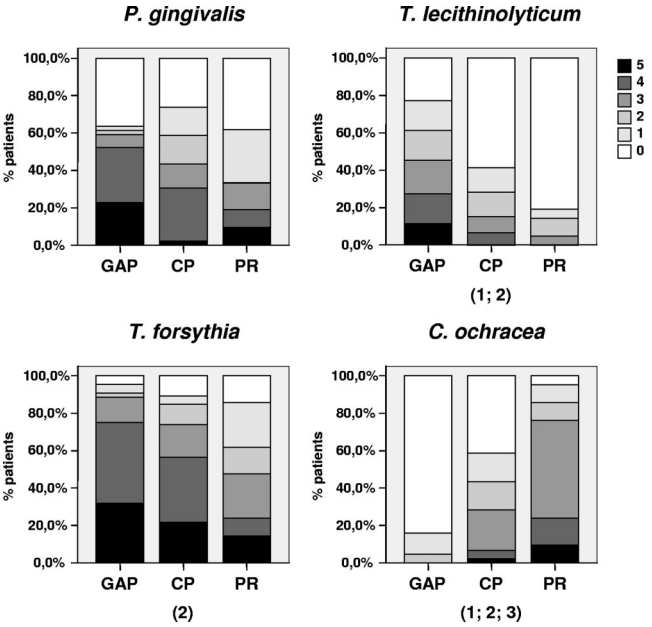

FIG. 3. Percentage of patients with 1, 2, 3, 4, or 5 of the sites colonized by target bacteria as revealed by dot blot analysis. Numbers in parentheses indicate statistical significances between the groups: 1, GAP versus CP; 2, GAP versus PR; and 3, CP versus PR, as determined with the Mann-Whitney U test. *P* values were adjusted for multiple comparisons (Bonferroni's correction).

The prevalences of the target species in the different groups are shown in Fig. 2. The number of individuals positive for a given species or phylotype varied considerably between the groups. Except for *C. ochracea*, the prevalence of most genospecies was highest in the GAP group, followed by the CP and PR groups (Fig. 2). Significant differences between the groups were found for *T. lecithinolyticum* ($P = 0.001$, GAP versus CP; $P < 0.001$, GAP versus PR), TRE II (*T. denticola*-like) ($P = 0.007$, GAP versus PR; $P = 0.002$, CP versus PR), *F. nucleatum* ($P = 0.001$, GAP versus PR), and *C. rectus* ($P = 0.013$, GAP versus PR). *A. actinomycetemcomitans* showed an overall significant difference ($P = 0.038$) but no significant differences between the groups in the post hoc test.

C. ochracea was detected significantly more often in PR subjects than in the two patient groups and significantly more often in CP patients than in GAP patients ($P < 0.001$, GAP versus CP and PR; $P = 0.002$, CP versus PR).

Number of positive sites per patient. To semiquantitatively assess the presence of the different species, we compared the number of positive sites per patient. Most putative periodontal pathogens were detected in more sites in both periodontitis groups, with numbers being highest in the GAP group (Fig. 3). The differences between the groups were statistically significant for *T. lecithinolyticum* ($P < 0.001$, GAP versus CP and PR), TRE II (*T. denticola*-like) ($P < 0.001$, GAP versus PR and CP versus PR; data not shown), *F. nucleatum* ($P < 0.001$, GAP versus PR; $P = 0.006$, CP versus PR; data not shown), *T. forsythia* ($P = 0.001$, GAP versus PR), *Fusobacteria* spp. ($P = 0.010$, GAP versus PR; data not shown), and *C. rectus* ($P = 0.009$, GAP versus PR; data not shown). *C. ochracea* showed significantly more positive sites in the PR group than in the two periodontitis groups ($P < 0.001$, GAP versus CP and PR and CP versus PR) (Fig. 3).

Presence of target bacteria depending on probing depths. The question arises of whether the greater prevalence of various bacteria in the periodontitis groups is due merely to the deeper pockets found in advanced periodontitis, resulting in more bacterial mass in the paper point sample. To evaluate the relationship between the existence of certain periodontal species and disease severity, logistic regression analysis adjusted for clustering on the subjects, as well as comparison of mean positive samples from pockets of different probing depths, was performed. The probability of occurrence of *C. ochracea* and *A. actinomycetemcomitans* showed no significant correlation with the probing depth. For all other putative periodontal pathogens, there was a positive correlation which was statistically significant ($P \leq 0.001$).

According to the inclusion criteria, the four deepest pockets had probing depths of ≥6 mm in the GAP group and <6 mm in the PR group. Comparison of mean positive samples was performed for all clinical groups only at probing depths of ≤3 mm. At probing depths of 4 to 5 mm, comparison was done only for the CP and PR groups, and at probing depths of 6 to 8 mm and ≥9 mm, only for the two periodontitis groups. At sites of 4 to 5 mm, the putative periodontal pathogens TRE II (*T. denticola*-like) and *F. nucleatum* were found significantly more often in CP patients than in PR subjects ($P < 0.001$ and $P = 0.027$, respectively; data not shown). At sites of 6 to 8 mm, *T. lecithinolyticum*, *A. actinomycetemcomitans* (data not shown), and *C. rectus* (data not shown) were detected significantly more often for GAP patients than for CP patients ($P =$

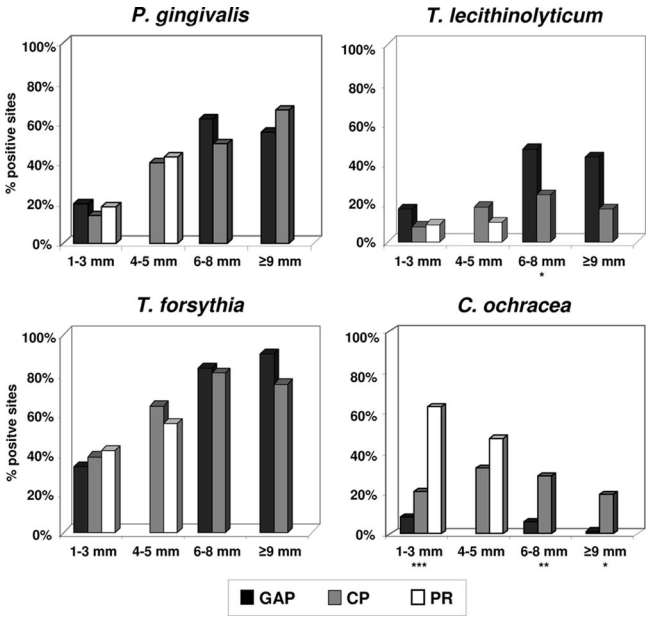

FIG. 4. Percentage of positive samples at different probing depths. According to the inclusion criteria, the four deepest pockets showed a probing depth of ≥6 mm in the GAP group and <6 mm in the PR group. Therefore, only the CP patient group and the PR group were compared at probing depths of 4 to 5 mm, and only the two periodontitis groups were compared at probing depths of 6 to 8 mm and ≥9 mm. Asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate significant differences as determined by analysis of variance. The mean percentages of positive sites were derived by averaging the positive sites of each species within a subject and then across subjects in the clinical groups.

0.013, 0.005, and 0.044, respectively) (Fig. 4). In contrast, *C. ochracea* was detected significantly more often in PR subjects than in the two patient groups at sites of ≤3 mm ($P < 0.001$) (Fig. 4). At sites of 6 to 8 mm and ≥9 mm, the differences were statistically significant between CP and GAP patients ($P = 0.003$ and 0.011, respectively) (Fig. 4).

DISCUSSION

It is widely accepted that the etiology of periodontitis is polymicrobial in nature (23). Worsening or improvement of periodontal status is accompanied by a shift in the bacterial composition of subgingival plaque (14). It has therefore been suggested that microbial testing can be used for diagnosis and to optimize periodontal therapy and assess its outcome, especially when treatment with antimicrobial drugs is considered. However, this strategy may be confounded, since initiation and progression of periodontal disease are influenced by the interaction of myriad genetic, environmental, host, and microbial factors (22, 24, 26, 32). Further, molecular studies reveal an unexpectedly high diversity of microorganisms whose relevance for initiation and progression of disease still remains to be investigated. Nevertheless, current microbiological testing mainly involves the classical suspected oral pathogens.

In the present study, we examined the prevalences of 10 periodontal bacterial species in 2 patient groups with advanced chronic or generalized aggressive periodontitis, as well as a periodontitis-resistant control group. We chose PCR of 16S rRNA gene fragments and subsequent dot blot hybridization as the detection method. Although quantitative analysis cannot be accurately performed with this approach, signal intensities in GAP samples appear much stronger than those in CP samples, even more so than those in PR samples (Fig. 1). In a semiquantitative approach, we compared the percentages of positive sites per patient. As expected, most putative periodontal pathogens were found more often in the periodontitis groups, especially the GAP group, than in the PR group (Fig. 3). The only significant differences in microbial prevalences between GAP and CP patients were seen for *T. lecithinolyticum* (Fig. 2).

Three widely accepted periodontal pathogens, *T. forsythia*, *P. gingivalis*, and *P. intermedia*, indeed showed no differences in prevalence between the three groups. This has also been shown by Kumar et al. (14) using quantitative 16S cloning and sequencing. These authors could not detect any significant association of *T. forsythia* and *P. gingivalis* with disease. However, these species represent only a small percentage of the total bacterial species, and the open-ended approach of their study may not be geared to detect an association of these species with disease. In another study, Kumar et al. demonstrated a significant association of both *T. forsythia* and *P. gingivalis*, as well as *T. denticola*, with chronic periodontitis, using PCR amplification of 16S rRNA genes (13). *T. forsythia*, *P. gingivalis*, and *T. denticola* are members of the so-called "red complex" based on checkerboard DNA-DNA hybridization of 13,000 plaque samples from 185 subjects (28). These three species are considered to be highly associated with advanced periodontitis. In the present study, the high prevalences of these species were confirmed in the periodontitis groups, but the PR group also showed very high prevalence rates (95%, 89%, and 86% for *T. forsythia*, 64%, 74%, and 62% for *P. gingivalis*, and 70%, 76%, and 67% for *P. intermedia* in GAP, CP, and PR groups, respectively, Fig. 2). The high prevalences of *P. gingivalis* and *T. forsythia* found here are similar to those in other studies examining aggressive periodontitis patients (12). Griffen and coworkers (8) found a comparable prevalence of *P. gingivalis* in periodontitis subjects using nested PCR analysis of samples from all teeth. However, the prevalence of *P. gingivalis* was significantly lower in age-matched healthy subjects. Although the mean age was higher, other inclusion criteria for the healthy control group were similar to those used in the present study. The authors concluded that *P. gingivalis* is highly associated with periodontitis, in accordance with results in other studies.

Most previous studies of the association of specific bacteria with periodontitis did not examine the influence of probing depth. We questioned whether the detection of a bacterial species may be related more to the depth of the sampled pocket than to a certain diagnosis. For most species in our study, we found that the probing depth had a much greater impact on the occurrence of the species than did the diagnosis. The prevalence of *P. gingivalis* was highly associated ($P < 0.001$) with pocket depth, as revealed by logistic regression analysis adjusted for clustering on the subjects.

Treponemes have previously been associated with periodontitis, and *T. denticola* especially has been suggested as a diagnostic marker (28). In the present investigation, treponemes

were more prevalent and were found in more sites in the periodontitis groups than in the control group. This was the case for both for *T. lecithinolyticum* and TRE II species, which include *T. denticola.* However, the difference was greatest for *T. lecithinolyticum*, as we have previously shown (21). Only *T. lecithinolyticum* showed significant differences between the two periodontitis groups. The differences between CP patients and the PR control group were not statistically significant, which again were highly significant for TRE II. This confirms the results of Kumar et al. (13), who compared the microbiota of chronic periodontitis patients with age-matched controls and detected *T. denticola* and to a lesser extent *T. lecithinolyticum* for significantly more diseased patients than controls.

A. actinomycetemcomitans has been closely associated with aggressive periodontitis, especially localized forms (previously known as localized juvenile periodontitis) (7, 34). It has been shown that the prevalence of *A. actinomycetemcomitans* in severe or refractory periodontitis seems to be inversely related to age. (26). However, Umeda et al. (32) found a positive correlation between age and the prevalence of *A. actinomycetemcomitans* in the subgingival space or saliva, using 16S rRNA PCR analysis. In the present study, the prevalence of *A. actinomycetemcomitans* is highest in the GAP patients, who are also the youngest, and lowest in the PR subjects, who are the oldest. The differences between the groups were statistically significant (Fig. 2). Logistic regression analysis showed that *A. actinomycetemcomitans* was the only "classical" putative periodontal pathogen tested whose presence did not correlate with probing depth. This is contrary to findings of Mombelli et al. (19) that the presence of *A. actinomycetemcomitans* is significantly associated with probing depth. However, these workers used culturing techniques that may not have detected low levels of the organism in shallow pockets. The detection frequency of *A. actinomycetemcomitans* was rather low for all subjects (36%, 20%, and 10% in GAP, CP, and PR subjects, respectively), which is in accordance with results in other studies analyzing samples from aggressive periodontitis patients (11, 12). Using DNA probe analysis of the four deepest pockets of patients, Haffajee and Socransky (9) found a 38% false-negative detection rate for species with low prevalences, such as *A. actinomycetemcomitans.* This finding questions the use of *A. actinomycetemcomitans* as an appropriate diagnostic marker.

In contrast to the suspected periodontal pathogens noted above, *C. ochracea* was significantly more prevalent and showed significantly more positive sites per patient in the control group than in the periodontitis groups. It was also significantly more prevalent in the CP than in the GAP patient group, independent of probing depth. This is consistent with results in other studies, which found that high levels of *C. ochracea* were related to a lower risk of disease progression (6, 10), suggesting that *C. ochracea* can be considered beneficial to the host. The detection of such beneficial species may be as important as detection of periodontal pathogens, and it has been suggested that recolonization of periodontal pockets with beneficial bacteria following scaling and root planing may be a useful clinical strategy (31). This approach has been studied in the gastrointestinal tract by use of probiotics or microbial replacement therapy (17) and deserves further investigation in the treatment of periodontitis.

Microbial testing has been advocated both for periodontal diagnosis and to discriminate between chronic and aggressive forms of periodontitis in order to better tailor the treatment approach. The results of this study indicate that testing for bacteria which are now presently targeted may not be sufficient. Most commercial tests include such putative pathogens as *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, *Fusobacterium* spp., and *P. intermedia*, which were included in this study. None of these organisms showed significant differences in prevalence between the two periodontitis groups. Their presence correlated with probing depth rather than diagnosis. The association of certain putative pathogens with severe periodontitis shown in many studies may be explained in part by deeper pockets in these patient groups. This view is consistent with the conclusion of Mombelli et al. (18) that the presence or absence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, or *C. rectus* cannot discriminate between subjects with aggressive or chronic periodontitis. Listgarten and Loomer (15) also questioned whether microbial identification should be considered as a strategy in the management of patients with periodontitis. They concluded that there was no strong evidence supporting the benefit of microbial testing, partly because of a lack of standardization among diagnostic laboratories.

In summary, the results of the present study indicate that currently recognized periodontal pathogens, such as *P. gingivalis*, *P. intermedia*, and *T. forsythia*, can be frequently detected in PR subjects and in both GAP and CP patients. However, the bacterial load seems to be lower in PR subjects, suggesting that these species are opportunistic pathogens. TRE II species (including *T. denticola*) are highly associated with GAP and CP patients compared to PR subjects, whereas *T. lecithinolyticum* was the only species showing significant differences both between GAP and CP patients and between GAP patients and PR subjects. Colonization by *C. ochracea* appears beneficial to the host, since it is associated with a stable periodontal condition, as evidenced by a high prevalence and frequency, independent of pocket depth, in subjects resistant to periodontitis. Therefore, we suggest *T. lecithinolyticum* be considered a diagnostic marker for GAP and *C. ochracea* an indicator of periodontal health.

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